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Immunoreactivation of Epstein-Barr Virus Due to Cytomegalovirus Primary Infection

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Serological diagnosis of herpes virus infections is hampered by concurrent expression of IgM for heterologous members of this virus family. To assess the frequency of such multiple diagnostic findings and to understand their etiology, we sought by using IgG, IgM, and IgG avidity test serodiagnoses for Epstein-Barr virus (EBV) among immunocompetent or immune-suppressed patients with well-documented cytomegalovirus (CMV) primary infection. Controls had primary infection by EBV or had acute septic or severe respiratory infection. Among EBV-seropositive patients with CMV primary infection, a large proportion (13/56, 23%) showed antibody profiles of EBV reactivation: seroconversion of VCA IgM and/or ⩾ fourfold rise of VCA IgG, together with high or intermediate avidity of VCA IgG. Most of the CMV patients with EBV serodiagnosis showed also diagnostic HHV-6 antibody rises. In contrast to the frequently occurring CMV-induced EBV immunoreactivation, EBV primary infections did not appear to induce immunoreactivations of CMV (0/22). Only one (2%) CMV patient had a significant varicella zoster virus (VZV) antibody rise. The studies show that CMV is a particularly active inducer of some, but not all, members of the herpes virus family and suggest that the in vivo interplay between CMV and EBV occurs unidirectionally. The high frequency of heterologous herpes virus immunoreactivations poses demands on laboratory diagnosis. ***J. Med. Virol. 56:186–191, 1998.***

KEY WORDS: avidity; diagnosis; herpes virus; recurrence; secondary infection

INTRODUCTION

Serological diagnosis of Epstein-Barr virus (EBV) infection is classically based on detection of heterophile antibodies and on measurement of antibody reactivity for various EBV antigens, including viral capsid antigen (VCA), the early antigens (EA), and members of the Epstein-Barr virus nuclear antigen (EBNA) family. Characteristic for EBV primary infection are positive VCA IgM and IgG results and lack of EBNA-1 antibodies [Henle et al., 1974]. EA IgG becomes detectable after VCA IgG and disappears within a few months [Henle et al., 1971]. During convalescence, VCA IgM antibodies usually disappear and the EBNA-1 test becomes positive, while VCA IgG persists for life. However, even in immunocompetent seropositive individuals EBNA-1 antibodies can sometimes remain negative [Lamy et al., 1982; Horwitz et al., 1985; Kampmann et al., 1993], and more often so in immunocompromised patients or in chronic mononucleosis [Henle et al., 1981; Miller et al., 1987; Kampmann et al., 1993].

A sign of viral reactivation can be the reappearance of VCA IgM antibodies, but VCA IgM in some patients remains detectable long after EBV infection even without a known reason [Schmitz et al., 1972; Sumaya, 1977]. Because EA IgG antibodies may reappear during EBV recurrence, they do not distinguish between primary and secondary infection [Henle et al., 1974, 1981]. Measurement of IgG avidity has been shown to be a powerful tool for differentiation of primary and secondary infections by various pathogens [Hedman et al., 1988, 1991, 1993; Blackburn et al., 1991]. Also in EBV serodiagnosis, avidity of VCA IgG or EA IgG separates primary and secondary infections both in immunocompetent and immunocompromised individuals [De Ory et al., 1993; Andersson et al., 1994; Vetter et al., 1994; Gray, 1995; Weissbrich, 1998].

Reactivations of EBV have been encountered among

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immunocompromised hosts such as organ transplant recipients or patients with HIV infection. However, to date no single commonly accepted serological criterion exists for EBV reactivation. Many different parameters have been used: rise of EA IgG or EA IgA titer [Hornef et al., 1995], seroconversion of EA IgM [Hornef et al., 1995], decrease of EBNA IgG [Quesnel et al., 1992; Taneichi et al., 1993; Hornef et al., 1995], presence of VCA IgM [Hornef et al., 1995], increase of VCA IgG [Rahman et al., 1991; Quesnel et al., 1992; Glaser et al., 1994], or simultaneous positivity to EA IgM and EBNA IgG [Obel et al., 1996]. The ZEBRA protein (BamHI Z EBV replication activator) controls the switch of EBV from a latent to a productive cycle [Countryman et al., 1985, 1987; Chevallier-Greco et al., 1986; Lieberman et al., 1986], and ZEBRA IgG has been proposed as a serological marker for EBV reactivation [Maréchal et al., 1993]. On the other hand, while ZEBRA antibodies in healthy EBV-seropositives are rarely detectable (2–4%), they not uncommonly (75–87%) occur in patients with nasopharyngeal carcinoma, but also during infectious mononucleosis (85%) [Joab et al., 1991; Mathew et al., 1994]. Furthermore, it seems that in the ZEBRA protein different B-cell epitopes associate with different EBV-associated diseases [Tedeschi et al., 1995].

In serological diagnosis of mononucleosis we have not infrequently encountered high-avidity VCA IgG antibodies, indicating past immunity, in EBV IgM-positive patients. Further studies of such patients have often revealed CMV primary infections (data not shown). Here, the frequency of diagnostic findings for EBV in patients with CMV primary infection has been assessed systematically and the clinical picture in such patients has been characterized.

MATERIALS AND METHODS

Patients

One hundred ninety-two samples of serum from 60 patients with serologically confirmed CMV primary infection were assayed. CMV primary infection was defined by seroconversion or ⩾ fourfold rise of CMV IgG of low avidity, together with detectable CMV-IgM antibodies. According to clinical data, 50 patients (age range 3–64 years, mean 31) with 154 sera were immunocompetent. The cardinal symptoms were: fever, lymphocytosis, fatigue, and headache (Table I). The mean period between the diagnostic samples was 37 days (S.D. 58). Of the 60 patients, 10 (38 sera) were organ graft recipients (2 heart, 3 liver, and 5 kidney) with posttransplant CMV primary infection (age range 17–61 years, mean 43). The serodiagnosis of CMV primary infection in the latter group was made 30 to 237 days (mean 105) after transplantation as indicated by the collection date of the first seropositive sample of each patient containing low-avidity CMV IgG. The mean period between diagnostic samples in this group was 90 days (S.D. 78).

All the samples were studied for EBV VCA IgM and IgG. Samples with diagnostic findings (seroconversion of VCA-IgM or ⩾ fourfold rise in VCA-IgG) were further examined for VCA IgG avidity, EA IgG, EBNA IgG, and HHV-6 IgG, as well as an immunofluorescence (IF) specificity control for nephropathia epidemica IgG (see below). For additional control, all the patients with CMV primary infection were studied for IgG antibodies against another latently infecting herpes virus, varicella zoster virus.

TABLE I. Clinical Picture in CMV Primary Infection With or Without EBV Immunoreactivation[a]

Symptom/finding	CMV with EBV immunoreactivation	CMV without EBV immunoreactivation
Fever	5.7 weeks	3.5 weeks
CRP > 10	86% (6/7)	87% (26/30)
ALT > 100	80% (4/5)	67% (16/24)
Fatigue	57% (4/7)	53% (16/30)
Headache	57% (4/7)	53% (16/30)
Sore throat	57% (4/7)[b]	17% (5/30)[b]
Eczema	43% (3/7)	23% (7/30)
Splenomegaly	43% (3/7)	27% (8/30)

[a]Patients with well-documented clinical data were included.
[b]The only symptom with a significant difference ($P < 0.05$).
CRP denotes C-reactive protein.
ALT denotes alanine aminotransferase.

Conversely, serum pairs from 22 patients (age range 1–42 years, mean 16) with EBV primary infection were studied for CMV IgM, IgG, and IgG avidity. The EBV primary infection was diagnosed by positive EBV VCA IgM together with low-avidity VCA IgG. The mean sample interval was 17 days (S.D. 12). All the serum samples described above had been referred for diagnostic purposes to the clinical virus laboratory of the Helsinki University Central Hospital.

As a separate control group, sera from 170 children (age range <1–15 years, mean 3.8) with acute septic or severe respiratory infection treated at Pediatric Hospital, University of Helsinki, were studied for VCA IgM, and follow-up samples of the IgM-positive sera were further studied for VCA IgG and IgG avidity. Serum pairs from these controls were also studied for HHV-6 IgG.

Serologic Methods

EBV VCA IgM was determined by an immunofluorescence assay (IFA) of Gull Laboratories (Salt Lake City, UT), in which rheumatoid factor interference was prevented by GullSORB IgG Inactivation Reagent (Gull). VCA IgG was measured by an EBV IgG enzyme immunoassay (EIA) (Gull) performed according to the manufacturer's instructions. EA IgG was measured by IFA (Gull). This test detects both diffuse (D) and restricted (R) components of the EBV EA complex. EBNA IgG was measured with anticomplement IFA (Gull) using as antigen Raji cells, which preferentially express EBNA-1. Heterophile antibodies were determined by the agglutination slide test Monosticon DRI-DOT (Organon Teknika, Boxtel, Netherlands). VCA IgG avidity was measured on EBV VCA EIA plates (Gull) by a protein-denaturing EIA employing endpoint titration of IgG [Hedman et al., 1993]. Briefly, the sera were diluted in PBS containing 0.05% Tween 20 (PBST) serially in fourfold steps from 1:40 to 1:10,240, or from 1:10

to 1:2,560, depending on the VCA IgG titer. After 1 hr incubation at 37°C, the wells containing the first (lowest) four dilutions were washed with 6 M urea in PBST and those with the last four (highest) dilutions with PBST alone. All wells were then treated for 30 min at 37°C with alkaline phosphatase-conjugated antihuman IgG followed by substrate. Two antibody titration curves were drawn for each sample: one of the urea-washed wells and the other of the PBST-washed wells. IgG avidity was calculated by the percent ratio of antibody titers (urea+/urea−) × 100. Avidity ⩽25% indicates primary infection; 25–40% is borderline; and ⩾40% indicates past immunity [Hedman et al., 1993; data not shown]. In three cases with controversial results, EBV-IgG avidity was measured by the Enzygnost EBV IgG kit (Behring Diagnostics, Marburg, Germany) containing an EBV antigen mix employing single dilutions of serum [Hedman et al., 1988] as described by Weissbrich [1998].

CMV IgM and CMV IgG were measured by indirect EIA (Labsystems, Helsinki, Finland) according to the manufacturer's instructions. CMV IgG avidity was measured with the CMV IgG-EIA kits (Labsystems) essentially as described above for VCA IgG avidity. CMV-IgG avidity ⩽20% indicates primary infection; 20–35% is borderline; and ⩾35% denotes past immunity (data not shown).

HHV-6 IgG was measured by in-house IFA by using infected HSB-2 cells as antigen as described previously [Linnavuori et al., 1992]. Nephropathia epidemica IgG was determined by IFA with Puumala virus-infected Vero E6 cells [Hedman et al., 1991]. The pattern of IgG fluorescence indicating acute infection or past immunity was evaluated and categorized as shown before [Vapalahti et al., 1995]. Varicella zoster IgG was measured by indirect EIA on polystyrene strips (Labsystems) coated with varicella zoster EIA antigen (Virion, Rüschlikon, Switzerland).

RESULTS

Among the 50 immunocompetent patients with CMV primary infection, 46 were EBV IgG (VCA)-seropositive and altogether 18/46 (38%) patients had VCA-IgM antibodies in one or more sera. In analysis of sequential samples from each patient, eight (17%) patients showed seroconversion of VCA IgM and/or ⩾ fourfold rise of VCA IgG, together with high or intermediate avidity of VCA IgG. Of the eight patients, six had ⩾ fourfold rises of VCA IgG and four had seroconversions of VCA IgM. All eight patients were EBNA IgG-seropositive; one of these showed a fourfold titer rise in EBNA IgG. EA IgG was detectable in six of the eight patients, with IgG titers ranging from 1:10 to 1:40. Of these eight patients, four had in their first samples high (>40%) VCA IgG avidity and four had borderline (25–40%) avidity. In one patient, the VCA IgG avidity decreased during follow-up, finally reaching a low level. However, with the EBV antigen mix, the IgG avidity of this patient remained constantly high, confirming that this patient had past immunity for EBV. Among these eight patients only one was barely and transiently positive for heterophile antibodies and another one had a borderline result; the remaining six patients had no detectable heterophile antibodies.

Interestingly, all eight patients with both a CMV and an EBV diagnosis had also high (⩾160) HHV-6 IgG titers; four had ⩾ fourfold titer rises in HHV-6 IgG. The EBV and HHV-6 antibody data are shown in Table II. Of these eight patients with EBV antibody rises, two had Puumala-virus IgG antibodies of past immunity; nonspecific antibodies against uninfected Vero E6 cells were not observed.

The clinical data of the patients with CMV primary infection-induced EBV antibody rises were compared with those having CMV primary infection without signs of EBV infection. All in all, the clinical picture in these two groups was similar. However, the patients with CMV-induced EBV immunoreactivation reported sore throat somewhat more frequently ($P = 0.04$) than those with CMV primary infection alone (Table I).

All 10 organ transplant recipients with CMV primary infection were EBV-seropositive; five showed serological pictures of EBV recurrence. Of these, three had ⩾ fourfold increases of VCA IgG and three had seroconversions of VCA IgM. Five patients had EBNA IgG; none showed decreasing titers, whereas one had a fourfold titer rise in EBNA IgG. EA IgG was detectable in 2/5 patients, of which four had in their first samples high (>40%) VCA IgG avidity and one had borderline (25–40%) avidity. Two patients showed decreases of VCA IgG avidity, whereas with the EBV antigen mix such avidity decreases were not seen. None of these five patients had heterophile antibodies.

Concomitant with their serological picture of EBV reactivation, all five patients demonstrated ⩾ fourfold titer rises in HHV-6 IgG. The EBV and HHV-6 antibody data of the five organ transplant recipients are summarized in Table III. One patient had past immunity for Puumala virus, while nonspecific Vero-cell antibodies were not seen.

Of the total 60 patients with CMV primary infection, 58 were varicella zoster virus-seropositive. The varicella zoster IgG antibodies rose significantly in only one patient. Among the 22 patients with EBV primary infection, 10 were CMV-seropositive, and none presented with a CMV serodiagnosis. Among the 170 controls with septic or severe respiratory infection, only two were EBV IgM-seropositive; both had low avidity of VCA-IgG, indicating EBV primary infection, whereas none showed a picture of EBV reactivation. Similarly, only 3/170 (2%) controls showed diagnostic HHV-6 IgG rises.

DISCUSSION

We demonstrated with widely used EBV antibody assays that a large proportion of patients with CMV primary infection showed antibody profiles of EBV recurrence: seroconversion of VCA IgM and/or ⩾ fourfold rise of VCA IgG, together with high (or intermediate) avidity of EBV IgG. Immunocompetent individuals and

TABLE II. EBV and HHV-6 Antibodies of Immunocompetent Patients With CMV Primary Infection-Induced EBV Immunoreactivation

Patient	Days of onset	VCA IgM	VCA IgG titer	VCA IgG avidity %	HHV-6 IgG titer[a]	Heterophile antibodies
1	7	+	160	29	320	–
	13	+	2,560	25	1,280	–
	27	+	20,480	28	ND	ND
2	–304	–	1,280	43	20	–
	18	+	1,280	46	10,240	–
	34	+	1,280	55	ND	ND
3	15	–	160	37	20	–
	24	–	160	40	ND	ND
	30	–	320	26	ND	ND
	51	–	1,280	14	640	–
4	16	–	2,560	54	ND	ND
	23	–	2,560	49	1,280	–
	27	+	2,560	52	ND	ND
	32	+	5,120	35	1,280	–
5	2	+	640	61	2,560	+/–
	37	+/–	5,120	52	2,560	+/–
	57	–	5,120	61	ND	–
6	8	–	1,280	39	160	+/–
	22	+	20,480	42	10,240	+
	30	+	20,480	41	ND	–
7	18	–	1,280	39	10,240	–
	21	+	2,560	59	ND	ND
	37	+	10,240	57	10,240	–
8	1	+	640	62	320	–
	9	–	1,280	42	ND	ND
	82	–	2,560	51	80	–

[a]ND denotes not determined.

TABLE III. EBV and HHV-6 Antibody Data of Organ Transplant Recipients With CMV Primary Infection-Induced EBV Immunoreactivation

Patient	Days from transplantation	VCA IgM	VCA IgG titer	VCA IgG avidity %	HHV-6 IgG titer[a]	Heterophile antibodies
9	58	–	160	38	20	–
	78	–	1,280	18	2,560	–
	332	–	320	31	10,240	–
10	–53	–	640	48	10	–
	–4	–	640	54	ND	ND
	2	–	640	44	ND	ND
	11	+	1,280	43	ND	ND
	16	+	1,280	35	ND	ND
	68	+	2,560	29	5,120	–
11	14	–	640	45	10	–
	57	+	1,280	37	160	–
	107	+	1,280	32	ND	ND
12	13	–	160	48	20	–
	102	–	1,280	9	2,560	–
13	22	–	640	46	40	–
	33	+	640	38	640	–

[a]ND denotes not determined.

transplant recipients differed in the frequency of CMV-associated EBV serodiagnosis (17% and 50%, respectively). In contrast, EBV primary infections reciprocally did not appear to induce immunoreactivations of CMV.

Theoretically, EBV antibody titer rises in CMV primary infection could be due to polyclonal B-cell stimulation, to antibody cross-reactivity, or to selective stimulation of memory B-cells, either with or without virus replication due to endogenous reactivation (or exogenous reinfection). Polyclonal antibody rises have been found long ago in CMV primary infection [Klemola et al., 1969], but these are usually of the IgM class, in contrast with the extensive titer rises of high-avidity VCA IgG found in many of our patients. Moreover, rising IgG for varicella zoster virus, another latently infecting herpes virus, was observed in only one of our CMV patients, which further argues against general immunoactivation as a cause of our findings. That cross-reactivity between the beta herpes virus CMV and the distantly related gamma herpes virus EBV would explain the high antibody titer rises is unlikely;

indeed, adsorption studies have shown no such cross-reactivity [Linde et al., 1990].

The vast majority of the patients with CMV-induced EBV immunoactivation showed also immunoreactivations for HHV-6. However, the specificity of the HHV-6 antibody rises is less clear-cut than that of the EBV serodiagnoses. CMV and HHV-6 show high sequence similarity [Efstathiou et al., 1988; Chou et al., 1992], and immunological cross-reactivities between these two viruses have been detected in some but not all antibody adsorption studies [Irving et al., 1990; Sutherland et al., 1991; Ward et al., 1991, 1993]. Kinetic differences have suggested that HHV-6 antibody rises in CMV patients would be at least partially virus species-specific [Irving et al., 1990]. In vitro virus replication studies [Flamand et al., 1993] have also supported the possibility that EBV could serve as a stimulatory cofactor for the induction of HHV-6 or vice versa.

Evidence is accumulating on virus–virus interactions in vivo. EBV reactivation by human immunodeficiency virus (HIV) has been documented by appearance of EBV particles in blood and secretions [Birx et al., 1986; Alsip et al., 1988; Diaz-Mitoma et al., 1990] and by the progressive increase of VCA IgG in relation to a decrease in CD 4 cell count [Quesnel et al., 1992]. Drouct et al. [1997] briefly reported signs of CMV and EBV coinfections in graft recipients and speculated that CMV could stimulate EBV replication and dissemination. Findings similarly suggestive of EBV activation (coexpression of EA IgM and EBNA IgG) have been observed in apparently immunocompetent [Färber et al., 1993] or immune-suppressed [Hornef et al., 1995] patients with CMV infection. Our data provide first unequivocal evidence for EBV immunoreactivations in a large proportion of patients with CMV primary infection. The most plausible explanation is stimulation of EBV-specific memory B-cells due to iterated EBV replication. However, definitive proof of this will only be obtained by quantitative detection of virus replication.

In support of earlier data [Hornef et al., 1995], among our immunocompetent CMV patients the additional EBV serodiagnosis neither augmented the general severity nor altered the pattern of the clinical picture. However, it was interesting to observe that sore throat was associated with CMV-induced EBV immunoreactivation significantly more often than with CMV primary infection alone. The number of transplant recipients studied was not sufficient for analysis of the clinical significance of CMV-induced EBV immunoreactivations during immunosuppression. Very recent studies show that HHV-6 increases the clinical severity of CMV reactivations [Dockrell et al., 1997].

The findings make it clear that, with widely used serological procedures a large number of CMV primary infections are being diagnosed as EBV infections. Such errors can have serious consequences during immune suppression or pregnancy, when CMV infections are health threatening. Furthermore, it can be inferred from these results that the criteria of EBV serodiagnosis need strengthening. Correct identification of the primary infectious herpes virus requires serological markers stronger than IgM detection or IgG rise. Consistent with earlier data, the findings showed the usefulness of IgG avidity determination in pointing out the primary infectious agent.

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